Universidade de Aveiro Departamento de Biologia
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Tiago André Azevedo dos Santos

Fish personality, memory, and learning hability – the effect of pharmaceuticals and abiotic factors

Personalidade, memória e capacidade de aprendizagem dos peixes – os efeitos de fármacos e factores abióticos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica dos Doutores Marcelino Miguel Guedes de Jesus Oliveira, Paula Inês Borralho Domingues e Miguel Maria Borges da Costa Guint Barbosa do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave comportamento, *Danio rerio*, personalidade animal, gemfibrozil, respostas bioquímicas

resumo

Nos últimos anos, a personalidade animal tem vindo a atrair a atenção da comunidade científica, estando neste momento a ser questionada a hipótese de da sua importância na sobrevivência e a evolução das espécies em situação de stress. De entre os diferentes potenciais agentes causadores de stress estão os contaminantes ambientais emergentes como, por exemplo, os fármacos. No entanto não existem muitos estudos de comportamento e personalidade dos peixes. Este trabalho visou aumentar o conhecimento de como fatores associados a personalidade podem influenciar a resposta a fármacos detetados no ambiente. Como organismo modelo foi escolhido o peixe zebra (*Danio rerio*), tendo sido selecionado organismos com 8 meses. Os animais foram separados, com base em resposta comportamentais, em duas classes, proativos e reativos. Após a separação, os organismos foram expostos, durante 96h a um fármaco humano, gemfibrozil, utilizado como regulador lipídico. Ao longo do ensaio experimental foram avaliados parâmetros comportamentais que permitiram avaliar a capacidade de resposta a estímulo, memória a adaptação. Ao fim de 96 h de exposição, parâmetros associados e stress oxidativo foram igualmente avaliados. Os peixes proativos percorreram uma maior distância que os peixes retroativos. Para além disso, os peixes retroativos expressaram níveis maiores de LPO que os peixes proativos, e os peixes retroativos do controlo e expostos à menor concentração de gemfibrozil expressaram mais LPO que os peixes retroativos expostos às concentrações maiores de gemfibrozil.

keywords behavior, *Danio rerio*, animal personality, gemfibrozil, biochemical responses

abstract

Over the last years, animal personality has been gaining a lot of attention from the scientific community, and, at the moment, it is being questioned its importance in survival and the evolution of species in stress situation. Between all of potential stress agents, there are emerging environmental contaminants, such as, pharmaceuticals. However, there are not many behavior and personality studies in fish. The present work aims to increase the knowledge of how factors associated with personality can influence the response to pharmaceuticals detected in the environment. For model organism, the zebrafish (*Danio rerio*) was chosen, and 8 months old organism were selected. Animals were separated, based on behavior responses, in two classes, bold and shy. After the selection, organisms were exposed, during 96h to a human drug, gemfibrozil, used as a lipid regulator. During the experimental assay, behavior parameters that allowed to evaluate the capacity of response to stimuli, and memory to adaptation were assessed. After 96h of exposure, associated parameters and oxidative stress were equally assessed. Bold fish traveled a bigger distance than shy fish. Furthermore, shy fish expressed higher LPO levels than bold fish, and fish from the control group, as well as, shy fish exposed to the lowest concentration of gemfibrozil expressed more LPO than shy fish exposed to the higher concentrations of gemfibrozil.

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1.Introduction

1.1. Behavior and personality

The different behaviors displayed in animals of the same species towards the same stimuli has lead researchers to believe that animals have different personalities, which affect their fitness (Briffa & Weiss, 2010). For that reason, it is important to know how personalities affect an animal's life.

Behavior can be defined as the response to stimuli. These responses are determinant in the definition of an organism personality, which refers to all of an individual's emotions, physiological and behavioral responses to changes in the environment (Castanheira et al., 2013). Personality can be defined as stable and long-term inter-individual behavior differences with intra-individual consistence (Carere & Locurto, 2011; Kazdin, 2000). These differences are caused by genetic and environmental influence, and permanently affect the phenotype of the individual (N. J. Dingemanse & Araya-Ajoy, 2015). As such, the study of personality focuses on understanding individual differences in particular personality characteristics, and understanding how different behaviors come together in an individual (Kazdin, 2000). Animal personality and human personality have been interpreted differently. In animals, individual variation in behavior has often been interpreted as random variation with no biological consequences, whereas in humans it is seen as meaningful and consistent personality traits, that affect the individual's health, survival rate, and with evolutionary consequences (Carere & Locurto, 2011). Nowadays, behavioral individual variations are starting to be seen as consistent throughout time, context in which they occur, environmental or social situation, and measure (Briffa & Weiss, 2010).

It has been observed in various studies that animals react in different ways to the same situation. Differences in behavior have a major influence in the survival rate of an animal and his conspecifics (Briffa, 2014), and can also have a significant evolutionary importance (Briffa & Weiss, 2010).

1.2. Personality theories, stress, and coping style

The new approach to animal personality has opened the door to several new theories that try to explain the variation of animal behavior and its evolutionary importance and conservation. Some of these theories are the adaptive theory, the quantitative genetic theory, and the bold-shy continuum.

According to the adaptive theory, individual differences in behavior, are explained by an adaption to the individual's physical and physiological features, as well as its interactions with the environment and social environment. These different behaviors are often correlated and consistent throughout the individual's life and through different contexts (N. J. Dingemanse & Araya-Ajoy, 2015; N. Dingemanse & Wolf, 2010; Wolf & Weissing, 2010).

According to quantitative genetic theory, not only does genetic expression influence an individual's behavior, but it can also affect its conspecifics behaviors. As such, the

interactions of an individual with his conspecifics in a social environment can act as an evolutionary selective pressure. Thus, the evolution of a group of individuals is influenced by not only the gene expression of each individual, but also the gene expression of his conspecifics (N. J. Dingemanse & Araya-Ajoy, 2015).

According to the bold-shy continuum, personality is characterized as a spectrum, being the extremes, bold and shy personalities, the main focus, due to the individuals having opposite behaviors. Bolder individuals have the benefit of outcompeting others for resources, but they also take more risks, compete more with their conspecifics, thus having a bigger physical strain, and are more susceptible for predation, as opposed to shy individuals, allowing these two different personality types to exist and survive in a population (Carter et al., 2013).

It is known that bold and shy individuals have different cortisol levels, thus having different reaction when exposed to stressful situations (Oswald et al., 2012). Stress is induced by the cognitive evaluation of an aversive stimulus or stressor (Koolhaas et al., 1999). Coping is the cognitive, behavioral, and physiological efforts to manage internal and/or external stressful situations (Koolhaas et al., 1999; Matud, 2004).

It is known that stress responses cause the creation of reactive oxygen species (ROS), which cause damage in cell membranes, through lipid peroxidation (LPO) (Şahin & Gümüşlü, 2004). Thus, the body produces antioxidants, such as CAT and GST, in order to neutralize ROS (Mejia-Carmona et al., 2015; Şahin & Gümüşlü, 2004).

1.3. Gemfibrozil as a pollutant

The increase of the world population coupled with the improvement of healthcare, as well, as the increase of life expectancy, lead to an increase in the use of pharmaceuticals. This growth of the pharmaceutical industry results in an increase of waste, which, alongside human and animal excretion of active metabolites, and the use of pharmaceuticals in agriculture, contributes to the accumulation of pharmaceutical byproducts in waste waters, that can pass through sewage treatment, and even reach other sources of water, like rivers and lakes (Henriques et al., 2016). The abundance of these contaminants has an unknown, but possibly severe, effect on the aquatic wildlife. Therefore the study of the effects these contaminants have on the wildlife is a major interest, as it may affect animal survival, evolution, and the ecosystem, and can have a negative effect on human health, as some of the animals affected are part of the human food chain (Henriques et al., 2016).

Gemfibrozil (GEM) is a fibric acid derivative with hypolipidemic effects. GEM is anti-dyslipidemic, and as such, it reduces fat levels in the bloodstream. It is used to reduce cholesterol and triglycerides in the bloodstream, it prevents heart disease in people with high cholesterol level in the blood (“Infarmed - INFARMED, I.P., 2014”).

GEM interacts with peroxisome proliferator-activated receptors (PPARalpha) resulting in PPAR alpha-mediated stimulation of fatty acid oxidation. This enhances triglyceride-rich

lipoprotein clearance and reduces the expression of apolipoprotein C-III (apoC-III). The reduction in hepatic production of apC-III results in subsequent reduction of serum levels of very-low-density-lipoprotein cholesterol (VLDL-C). Furthermore, gemfibrozil-mediated PPARalpha stimulation of apoA-I and apoA-II expression results in an increase in high-density lipoprotein cholesterol (HDL-C) (Bruni et al., 2005; Honkalammi et al., 2012).
GEM has been found in aquatic environments, in concentrations of 19 µg/L in wastewater effluents (Fang et al., 2012). Furthermore, GEM has been found to influence locomotor behavior of zebrafish embryos (Henriques et al., 2016), as well as, increasing cortisol levels in *Sparus aurata* (Teles et al., 2016), justifying further studies on the effects of this pharmaceutical in fish.

1.4. Zebrafish, a model organism for behavior

The zebrafish (*Danio rerio*) (Figure 1) is a small fish (2.5 cm to 4 cm long) native to Southeast Asia, that has been trending in biomedical research. Zebrafish have started to be used as a model organism in the 1960s and since then it has become a model organism in many areas, such as, genetics, development, behavior, and toxicology (Kalueff et al., 2014) due to several characteristics of this species. Their dimensions allow them to be housed in large numbers in a small space, they are low maintenance, being significantly cheaper than housing and maintaining mice. They are easy to breed, generate a large number of offspring, have a rapid development, and live for a relatively long time (3-5 years). Furthermore, eggs and larvae are transparent, allowing to observe embryogenesis states and internal malformations without having the need to sacrifice them. Not only that, but its genome is already sequenced and well characterized, they have a significant physiological and genetic homology to humans (Kalueff et al., 2014).
Its popularity in neuropharmacological and behavioral studies comes from the homology of its Central Nervous System macro-organization and histology to humans and other mammals, as well as, similarity to all the major human neurotransmitters, their receptors, transporters, and enzymes of synthesis and metabolism (Kalueff et al., 2014).

Figure 1 - Adult zebrafish.

1.5. Aims

The aim of this study is to assess if personality, based on the bold-shy continuum, has an important role in the response to GEM and light/dark stimuli. For this, behavior responses were evaluated during 96h of GEM exposition, and biochemical responses were evaluated after 96h exposition.

2. Materials and methods

2.1. Test organism

This study used adult zebrafish (8 months old) provided by the facilities at the Department of Biology, University of Aveiro. The fish were maintained in carbon-filtered water, complemented with salt, "Instant Ocean Synthetic Sea Salt" at 27.0 ± 1 ˚C and exposed to a photoperiod cycle of 14:10 light: dark. Conductivity was kept at 750 ± 50 µS/cm, pH at 7.5 ± 0.5 and dissolved oxygen above 95% saturation. The fish were fed daily with the commercial artificial diet GEMMA Micro 500. Throughout the test, fish were maintained at the same temperature and photoperiod conditions.

2.2. Sorting of fish according to personality trait

To sort organisms, based on personality traits, a novel environment test was used. Randomly selected fish were placed in a habituation tank (40.6 cm x 26cm x19.5 cm) and allowed to acclimate for 30 min. After this period, 9 fish were transferred to the sorting tank (with the same dimensions as the habituation tank) which had two differently sized compartments (the smaller compartment was roughly one third of the size of the tank), divided by a wall with a small hole and an opening mechanism (Fig. 2). Fish were placed in the smaller compartment and allowed to explore this area for 15-minutes. After this period, the hatch was opened for 15 minutes, and fish allowed to pass to the bigger compartment. After this period, the hatch was closed and the fish that passed through the hatch were transferred to a tank labelled bold. The remaining fish, stayed an additional 15-minute habituation period. Finally, the hatch was opened for 15 more minutes as described above. The fish that passed through the hatch were labeled as intermediate and those that remained in the same compartment were labeled as shy.

2.3. Fish exposure to GEM

All reagents used were analytical grade and acquired from Sigma-Aldrich. GEM (≥98%) was purchased from TCI.

Fish (bold and shy) were individually exposed during 96h in aerated rectangular boxes (13 cm x 8cm x 5 cm) with 10 replicates per experimental condition, to a concentration range of GEM: a control solution (0), 0.015 mg/L, found in waste water effluents (Fang et al., 2012), 0.15 mg/L, which is an intermediate concentration to connect the lowest and highest concentration by a factor of ten, and 1.5 mg/L, reported to increase plasma cortisol levels in *S. aurata* (Teles et al., 2016). GEM solutions were prepared in fish water, and 300mL of solution was used for each box.

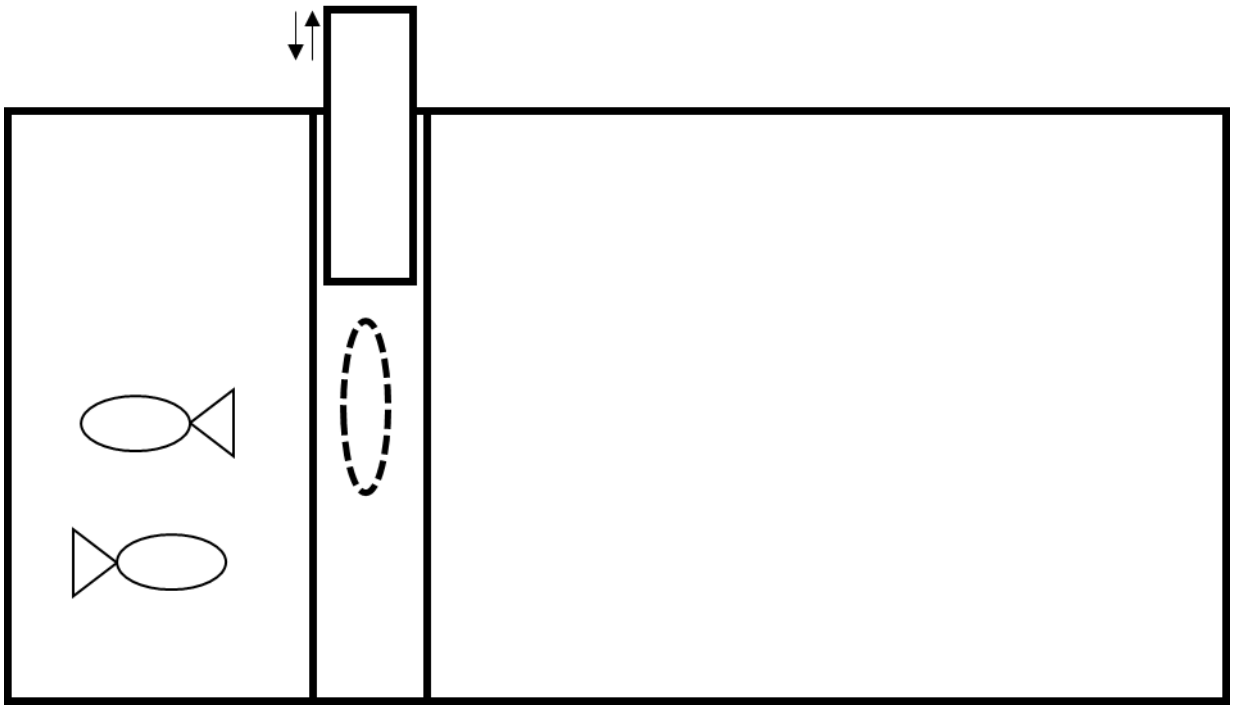

Figure 2 - Test tank to assess exploratory behavior. Fish were kept in the smaller compartment of the tank for 15-minutes, after this time the hatch was opened and fish were allowed to roam for 15-minutes, and then the hatch was closed. Fish that passed through are considered bold, the other fish stayed an additional 15-minutes, after this time, the hatch was opened again for 15 more minutes. Fish that remained in the smaller compartment of the tank were considered shy, and the others intermediate.

2.4. Behavior Biomarkers

Zebralab (Viewpoint Life sciences, Lyon, France) was used to track fish movement in light and dark conditions. Fish were place in the Zebrabox (component of the Zebralab) to study behavioral effects on bold and shy fish after GEM exposure using light/dark stimulus. To assess the active avoidance of the center of the tank (thigmotaxis) (Schnörr, Steenbergen, Richardson, & Champagne, 2012), two areas of the tank were defined as outside and inside (Fig. 3). Fish movements were tracked in the inside and outside areas, as well as, the time spent in each one, during six minutes in interchangeable light and dark periods, of one minute each. In this test, a change to a dark period is presented as a stressful event that may cause an increase in movement as well as a thigmotaxic response.

The fish behavior was measured after 3, 24, 48, 72, and 96h exposure. The test was performed every day at the same hour, during the afternoon, and the bold and shy fish were tested intercalated to avoid any differences of behavior potentially associated with the time of day.

After the 96h reading fish were sacrificed with over anesthesia (MS 222), and liver was sampled and stored at -80 ˚C until further processing.

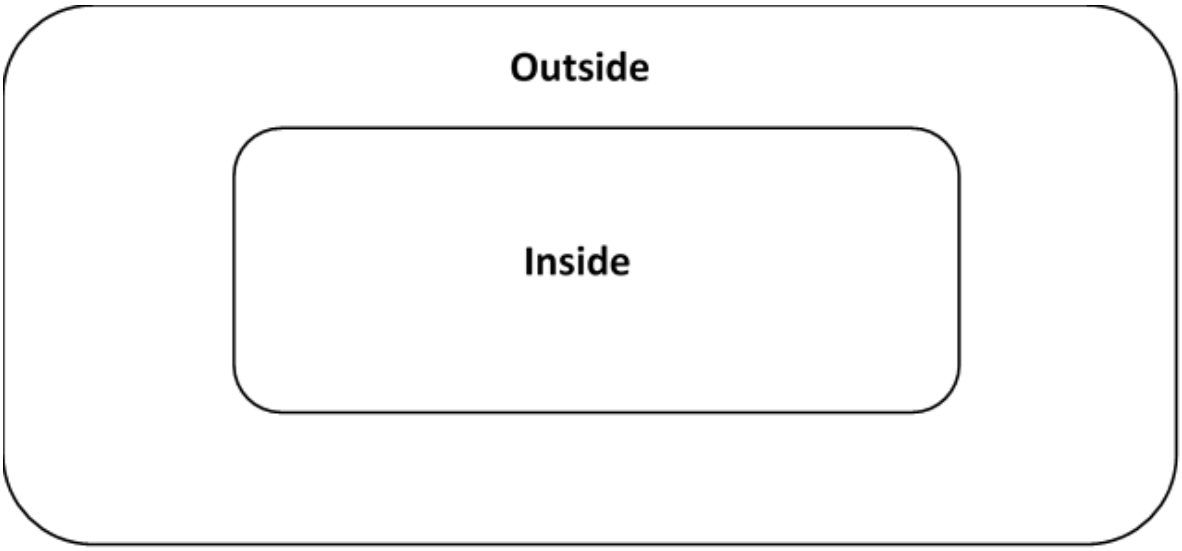

Figure 3 - Outside and inside area of the box that were considered by the Zebralab software, in order to assess thigmotaxis.

2.5. Biochemical Biomarkers

The liver samples were homogenized in phosphate buffer (0.1 M, pH=7.4), and then divided in aliquots for lipid peroxidation (LPO) and for PMS isolation (13400 *g* for 20 minutes at 4˚C) for determination of glutathione S-transferase (GST) and catalase (CAT).

LPO was measured using the method described by Ohkawa et al. (1979) adapted to microplate (M. Oliveira et al., 2009), and expressed as nmol of thiobarbituric acid reactive substance (TBARS) formed per mg of protein.

GST was determined by the conjugation of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm at 25˚C, and expressed as nmol of CDNB formed per minute per mg of protein, measured using the methodology of Habig & Jakoby (1981) adapted to microplate (Frasco & Guilhermino, 2003).

CAT was measured using the method described by Claiborne (1985), determining the consumption of the H_2O_2 substrate at 240 nm.

Activity of the enzymes was normalized by the protein content of the samples which was determined by the method described by Bradford (1976) applied to microplate at 25ºC.

2.6. Statistical Analysis

All the graphs and statistical analysis were put together using the SigmaPlot software. A two-way analysis of variance was used to infer statistical significance (set at $p<0.05$), defining personality and GEM as parameters. Normality was tested using the Shapiro-Wilk normality test. When normality failed, a Holm-Sidak test was used for an all pairwise multiple comparison procedure.

3. Results

3.1. Behavior Biomarkers

From the data of the behavior test, there were no significant differences observed 3h after exposure to GEM.

When the total distance traveled was analyzed, it can be observed, during every test day, a slight habituation of bold and shy fish, as it can be seen in Figures 4 – A, and 4 – B, for the 96h measurement. There were no significant differences between GEM concentrations, nor during light periods (60s, 180s, 300s). During the dark periods (120s, 240s, 360s) bold fish travel a significantly longer distance than shy fish, in the 24h ($p=0.030$), 48h ($p=0.027$), 72h ($p=0.008$), and 96h ($p=0.006$) measurements, as it can be seen in Annex-1, and for the dark period of the 96h measurement, in Figure 5 – A.

When analyzing the percentage of the distance traveled in the outside area, it was observed that, in dark periods fish travel more on the outside area than during light periods, as exemplified in Figures 4 – C, and 4 – D, for the 96h measurement. There were no significant differences between bold and shy personalities, nor during light periods. Fish from control group traveled significantly more than fish exposed to 0.15mg/L of GEM, during the dark period of the 48h measurement ($p=0.049$). Fish exposed to the 0.15 mg/L concentration traveled significantly less in the outside area of the box when compared to the fish exposed to the 1.5 mg/L concentration, during the dark periods of the 24h ($p=0.011$), 48h ($p<0.001$), 72h ($p=0.036$), 96h ($p=0.036$) measurements, as observed in Annex-2, and for the dark period of the 96h measurement in Figure 5 – B.

When analyzing the percentage of the time spent in the outside area, it can be observed that there were no significant differences in between bold and shy fish from the control group, and between fish from the control group and fish exposed to GEM. Fish exposed to 0.15 mg/L of GEM spent significantly less time in the outside area of the box compared to fish exposed to 1.5 mg/L of GEM, during the dark periods of the 24h ($p=0.009$) and 48h ($p=0.003$) measurements. During the dark periods of the 72h measurement, bold fish exposed to 1.5 mg/L GEM spent significantly more time in the outside area than shy fish exposed to the same concentration ($p=0.050$). During the light periods of the 72h measurement, shy fish exposed to the 0.015 mg/L of GEM spent significantly more time in the outside area than bold fish exposed to the same concentration ($p=0.043$), furthermore, shy fish exposed to 0.0 15 mg/L of GEM spent significantly more time in the outside area than shy fish exposed to 1.5 mg/L ($p=0.025$). There were no significant differences 96h after exposure, as observed in Annex-3, and for the dark period of the 96h measurement in Figure 5-C.

3.2. Biochemical biomarkers

When analyzing CAT levels (Fig. 6 – A), bold fish from the control expressed significantly more CAT than shy fish from the same group ($p<0.001$). Bold fish also expressed significantly more CAT than bold fish exposed to 0.015 ($p<0.001$), 0.15 ($p=0.002$), and 1.5 ($p<0.001$) mg/L GEM concentration. There were no further significant differences.

When analyzing GST levels (Fig. 6 – B), shy fish expressed significantly more GST than bold fish ($p=0.045$). There were no further significant differences.

When analyzing LPO levels (Fig. 6 – C), shy fish expressed significantly more LPO than bold fish in 0 ($p<0.001$), 0.015 ($p<0.001$), 0.15 ($p=0.019$), and 1.5 ($p=0.023$) mg/L of GEM exposure. Shy fish from the control group expressed significantly more LPO than shy fish exposed to 0.15 mg/L of GEM ($p=0.019$). Shy fish exposed to 0.015 mg/L of GEM expressed significantly more LPO than shy fish exposed to 0.15 mg/L of GEM ($p=0.003$). Furthermore, shy fish exposed to 0.015 mg/L of GEM expressed significantly more LPO than fish exposed to the 1.5 mg/L of GEM ($p=0.039$).

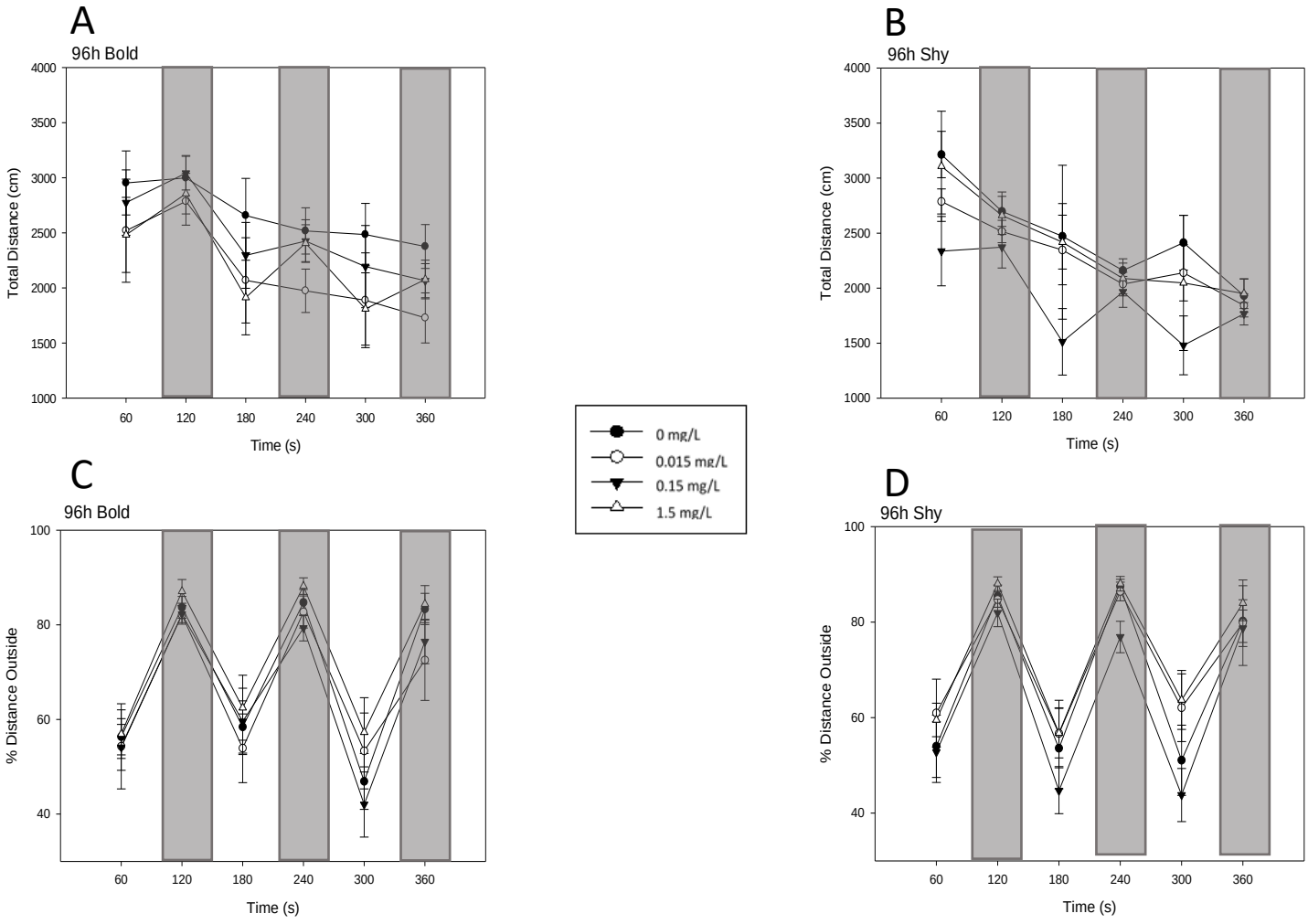

Figure 4 - Total Distance traveled 96h after exposure with GEM in bold (A) and shy (B) fish, and percentage of the distance traveled in the outside area 96h after GEM exposure in both bold (C) and shy (D) fish

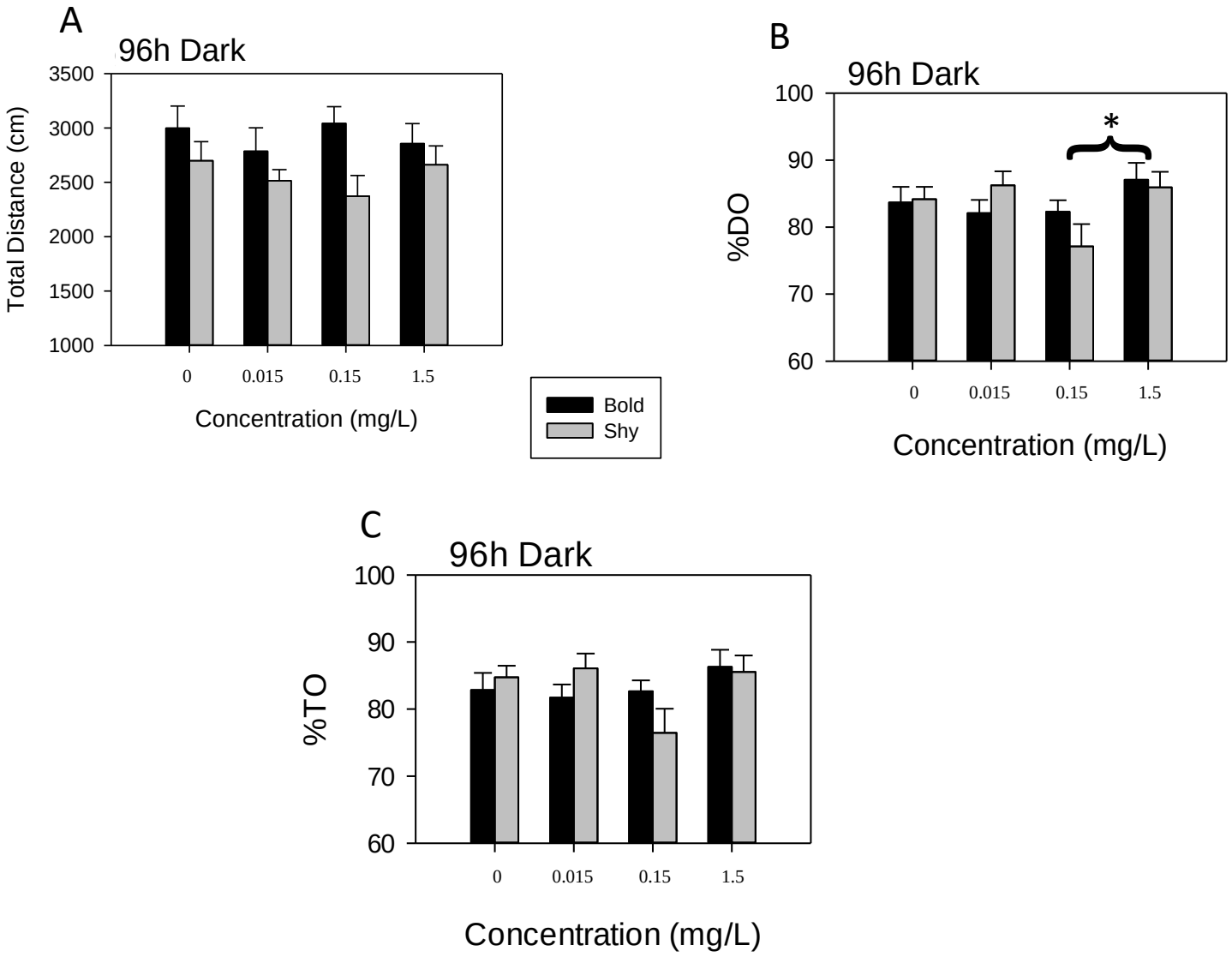

Figure 5 - Behavior of shy and bold fish after 96h exposure to GEM. Total distance traveled (A), percentage of the distance traveled in the outside area of the box (B), and percentage of the time spent in the outside area of the box

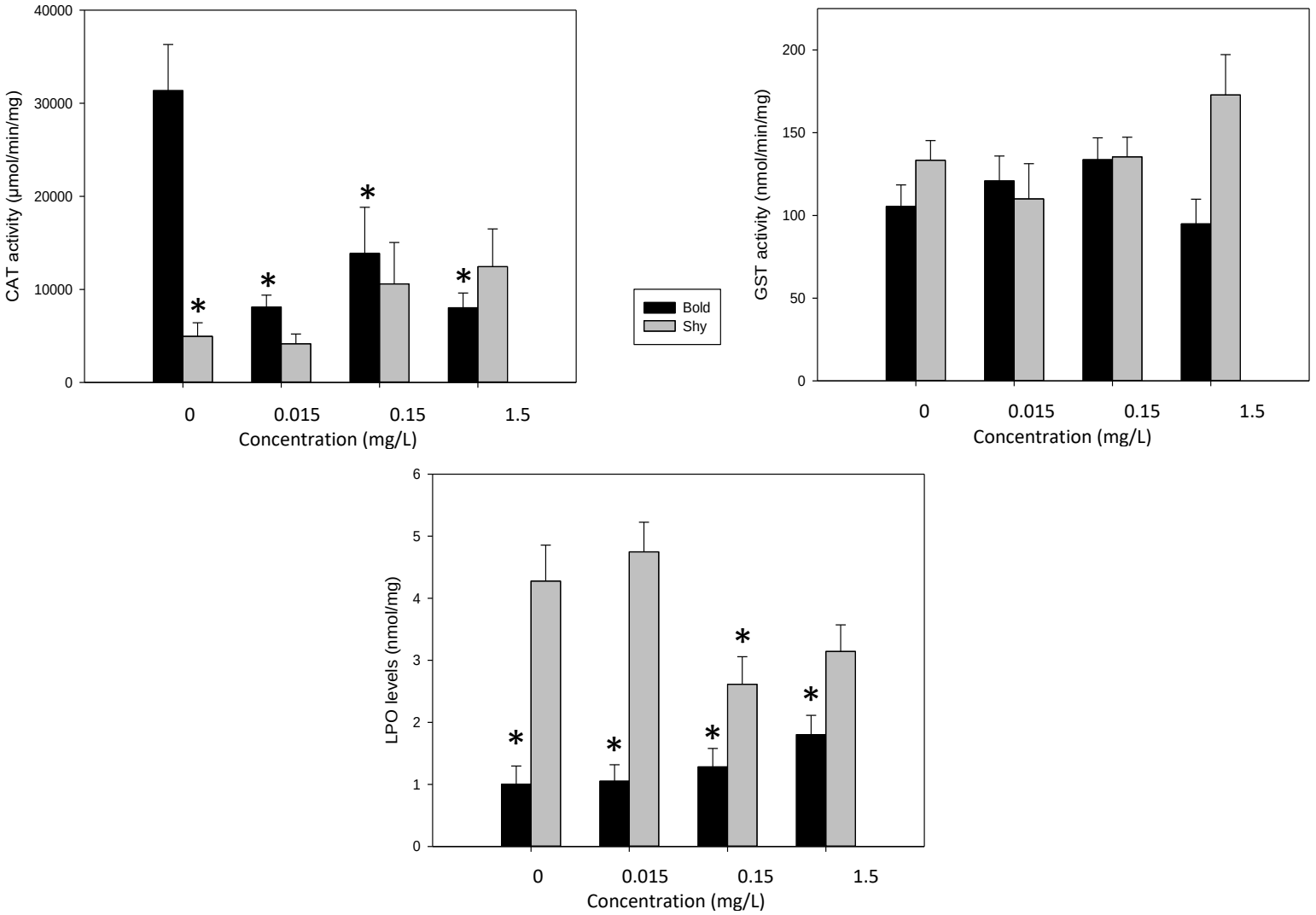

Figure 6 - Levels of CAT (A), GST (B), and LPO (C) expressed in bold and shy fish exposed to different GEM concentrations

4. Discussion

4.1. Behavior Biomarkers

Drug pollution is a major concern in aquatic environments, and has severe consequences in aquatic ecosystems. It is important to learn in which way different drugs affect an organism, and, as personality plays a major role in an individual and its species survival and evolution, it is a subject of interest for this field of study.

The results from the light/dark behavior test, in the dark, fish travel more in the outside area of the box, showing thigmotactic behavior (fish tend to travel more on the edges of the box). During the 3h measurement there were no significant differences, this may be due to the fish still being in a habituation period when the test started, as the fish are social and are not used to being in individual boxes, in addition to that, GEM still had not had a visible effect

In this study, bold fish travel significantly longer distances than shy fish, meaning that bold fish are more active, as seen in previous studies with *Sparus aurata* (Castanheira et al., 2013). In the control group and lowest concentration of GEM, shy fish tend to travel and spend more time in the outside area of the box, during both light and dark periods.

4.2. Biochemical biomarkers

Bold fish from the control group significantly more CAT than all GEM concentrations, and the control group of shy fish. CAT responds to ROS levels and is mainly used to neutralize hydrogen peroxide (R. Oliveira et al., 2016). CAT results were not consistent with other studies in zebrafish (R. Oliveira et al., 2016).

Shy fish express significantly more GST than bold fish. This enzyme is used to neutralize xenobiotics (Domingues et al., 2010), as such, xenobiotic may be found more in shy than in bold fish.

In all GEM concentrations, shy fish expressed significantly more LPO levels than bold fish. In shy fish, 0 and 0.015 GEM concentrations were significantly different from 0.15, and 0.015 was significantly different from 1.5. Shy fish exposed to higher GEM concentrations express lower levels of LPO, however, the higher the concentration in bold fish, the higher their levels of LPO expression.

Both LPO and GST were expressed more in shy fish, these fish may be more prone to suffer from oxidative stress than bold fish.

4.3. Final Remarks

In conclusion, it was detected that bold fish are more active than shy fish, and that GEM affects thigmotactic behavior. The results from CAT were not consensual with the rest of the results. According to LPO and GST levels, shy fish seem to suffer more from oxidative stress than bold fish. It seems that bold fish tend to be more stressed when exposed to higher concentrations of GEM, and shy fish tend to be more relaxed when exposed to these concentrations.

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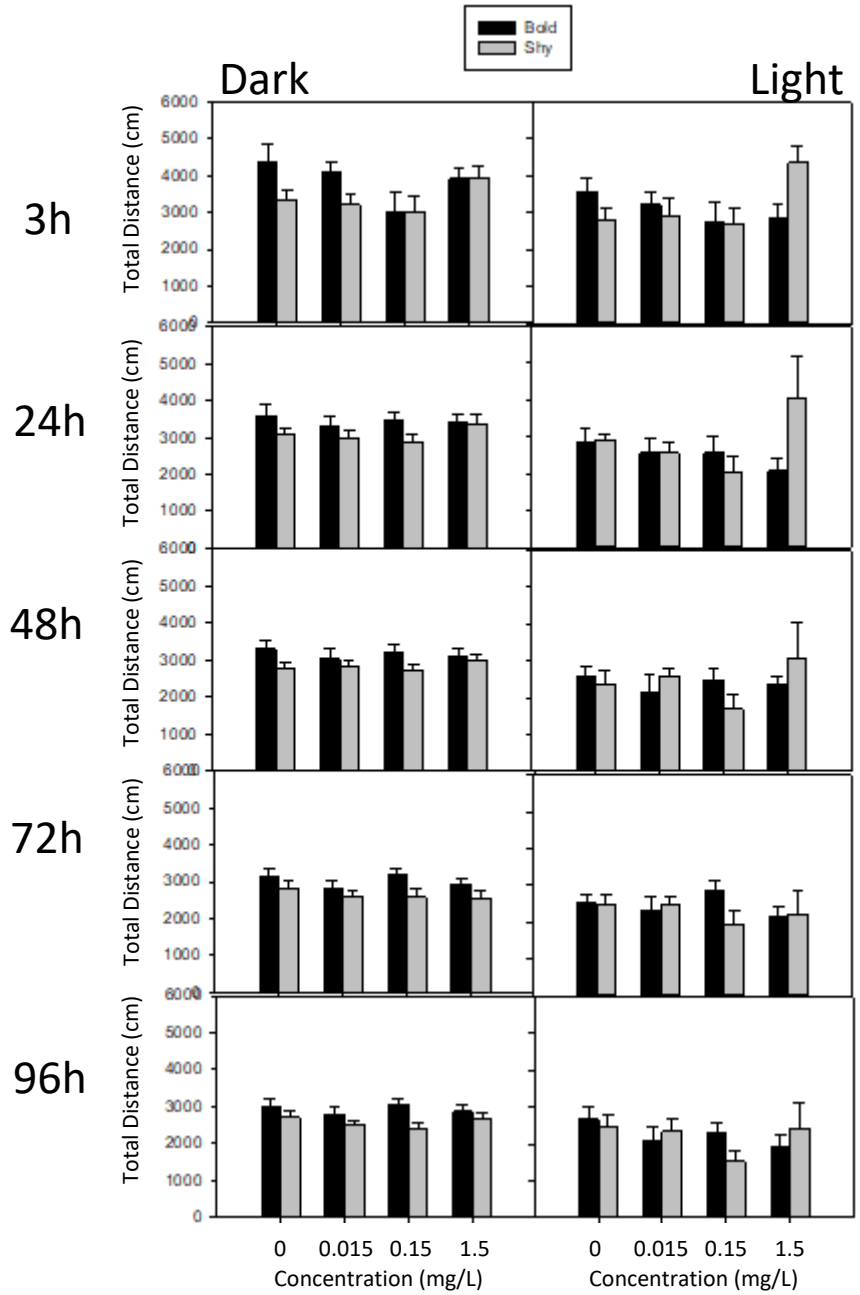
Annex

Annex-1


Bold
Shy
Dark
Light
3h
24h
48h
72h
96h
Total Distance (cm)
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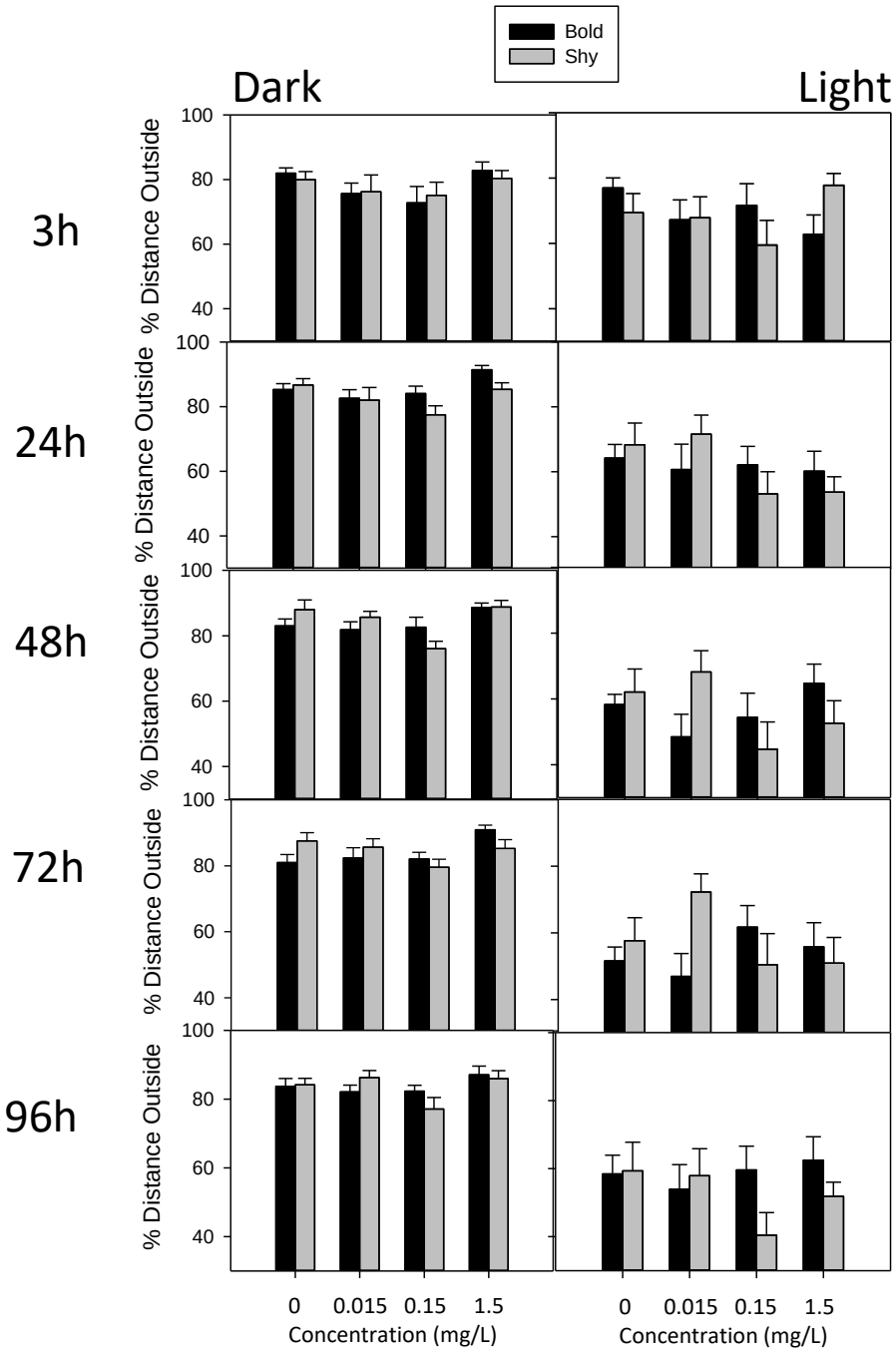
2000
3000
4000
5000
6000
0
0.015
0.15
1.5
Concentration (mg/L)

Annex-2


Bold
Shy
Dark
Light
3h
24h
48h
72h
96h
% Distance Outside
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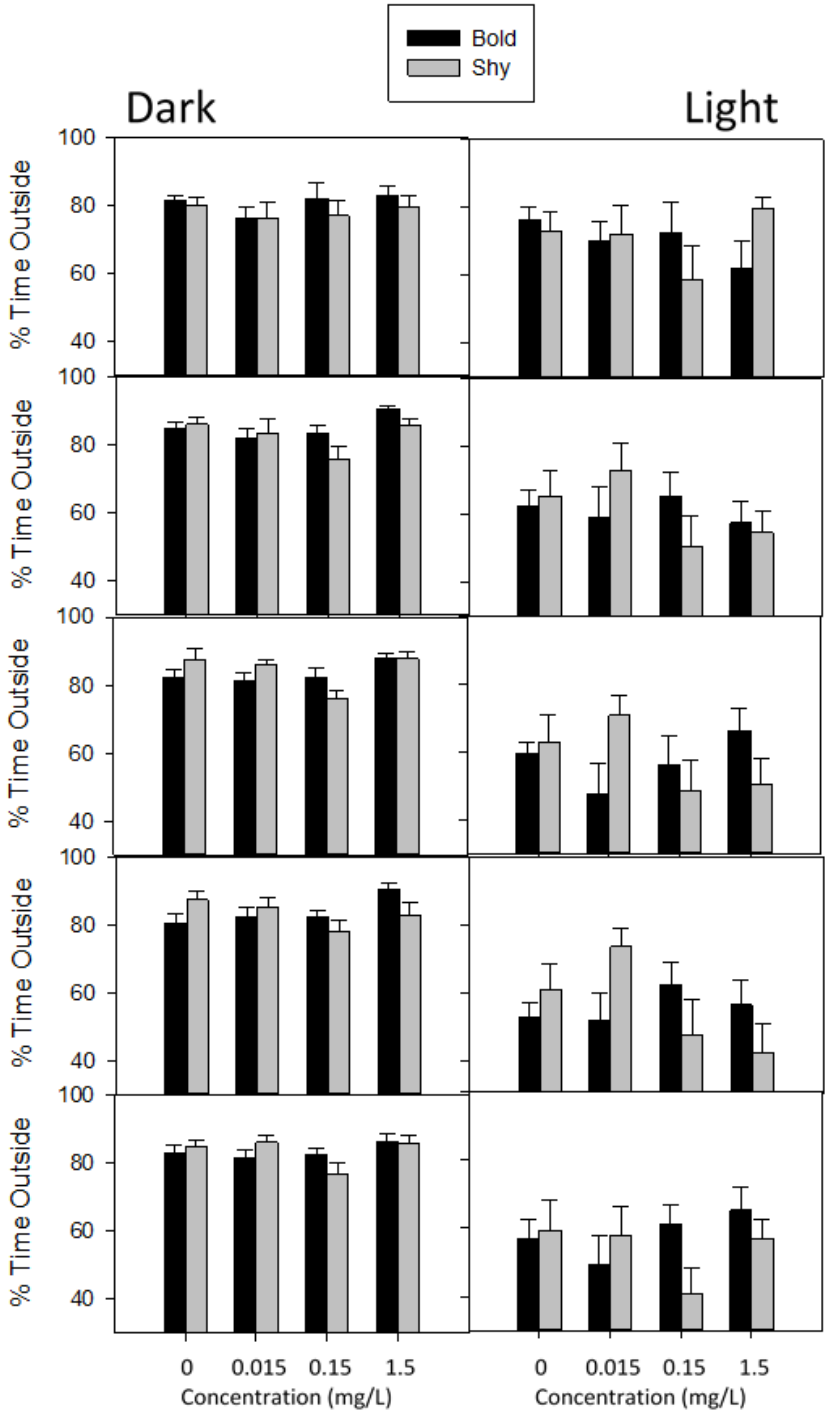
60
40
0
0.015
0.15
1.5
Concentration (mg/L)

Annex-3


Bold
Shy
Dark
Light
% Time Outside
100
80
60
40
0
0.015
0.15
1.5
Concentration (mg/L)